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Supporting Information

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Ribose 2'-F Labeling: A General Approach for the Identification of RNA Binders by ^{19}F-NMR Spectroscopy**

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1. Methods

Oligonucleotide synthesis

Cyanoethylphosphoramidites of 2'-*F* adenosine, 2'-*F* cytidine, 2'-*F* guanosine, and 2'-*F* uridine, were obtained from GlenResearch, Sterling, VA. All oligoribonucleosides with site-specific 2'-*F* labels were synthesized based on the 2'-*O*-TOM methodology on CPG supports on a Pharmacia Gene Assembler Special. 2'-*F* modified nucleoside cyanoethylphosphoramidites were coupled using standard synthesis protocol.

Detritylation (2.0 min): dichloroacetic acid/1,2-dichloroethane (4/96); coupling (3.0 min): phosphoramidites/acetonitrile (0.1 M x 120 µL) were activated by benzylthiotetrazole/acetonitrile (0.30 M x 360 µL); capping (2 x 0.4 min): A: Ac_2O/*sym*-collidine/acetonitrile (2/3/5), B: 4-(dimethylamino)pyridine/acetonitrile (0.5 M), A/B = 1/1; oxidation (1.0 min): I_2 (10 mM) in acetonitrile/*sym*-collidine/H_2O (10/1/5). Amidite solutions, tetrazole solutions, and aceonitrile were dried over activated molecular sieves overnight. All sequences were synthesized trityl-off.

Deprotection and cleavage of 2'-*F* modified oligonucleotides from solid support were achieved under standard conditions (CH_3NH_2 in EtOH, 8 M, 650 µL and CH_3NH_2 in water, 40 %, 650 µL, 6 h, room temperature). 2'-*O*-silyl-ethers were removed by treatment with tetrabutylammonium fluoride trihydrate in anhydrous THF (1 M, 950 µL) for at least 12 hours at room temperature. After addition of 950 µL triethylammonium acetate buffer (pH 6.8) the volume was reduced to approximately 1 mL and directly applied on a HiPrep 26/10 desalting column (Amersham Biosciences). The product was eluted with water and subsequently evaporated to dryness.

Further purification steps were carried out on a semipreparative Dionex DNAPac column at 80 °C (flow rate 2 mL min^{-1}; A 25 mM Tris.HCl, 6 M urea in water, pH 8.0; B 25 mM Tris.HCl, 6 M urea in water, 500 mM sodium perchlorate, pH 8.0). Fractions containing the purified oligonucleotide were desalted by loading on a C18 SePak cartridge (Waters/Millipore), followed by elution with 100 mM triethylammonium bicarbonate buffer, and then water/acetonitrile (1/1, v/v). Combined fractions of the oligonucleotide were lyophilized to dryness. The molecular weights of the synthesized oligoribonucleotides were confirmed by ESI mass spectrometry.

Thermal denaturation studies

Absorbance *versus* temperature profiles were recorded at 250 nm, 260 nm, 270 nm, and 280 nm on a Cary-100 spectrophotometer equipped with a multiple cell holder and a Peltier temperature-control device. Data were collected after a complete cooling and heating cycle at a rate of 0.7 °C/minute. Melting transitions were reversible and essentially the same with respect to the four different wavelengths.

Sample preparation

Oligonucleotides (triethylammonium salts) were lyophilized to dryness, dissolved in the corresponding buffer from stock solutions and subsequently degassed. A layer of silicon oil was placed on the surface of the solution.

Fluorescence Spectroscopy

Fluorescence spectra were acquired on a Varian Cary Ecplise fluorimeter equipped with a multiple cell holder and a Peltier temperature-control device. Time dependent data was collected with a excitation wavelength of 450 nm and fluorescence intensity was monitored at 530 nm.

Sample preparation

Oligonucleotides (triethylammonium salts) were lyophilized to dryness, dissolved in the corresponding buffer from stock solutions and subsequently degassed by sonication.

NMR spectroscopy

^{1}H NMR Spectroscopy

^{1}H-NMR-spectra were recorded on a Varian Inova 500 MHz NMR spectrometer equipped with a 5 mm *Indirect Detection PFG* probe applying a selective excitation refocusing sequence employing selective pulses shaped according to the G4 (excitation;[1] 2.62 ms, rf amplitude 1.74 kHz) or REBURP (refocusing;[2] 1.4 ms, rf amplitude 4.47 kHz) profile, respectively. Both shaped pulses were centered at 13 ppm.

^{19}F-NMR spectroscopy

^{19}F-NMR spectra without ^{1}H-decoupling were recorded at a frequency of 470.3 MHz on the same instrumentation (Varian Inova 500 MHz). Typical experimental parameters were chosen as follows: spectral width 7.5 kHz, ^{19}F excitation pulse 12 µs, acquisition time 2 s, relaxation delay 2 s, number of scans 2048. Time domain data were processed with an exponential window function using a line broadening factor of 12-30 Hz.

^{19}F-NMR spectra with ^{1}H-decoupling were recorded at a frequency of 376.5 MHz on a Bruker Avance DRX 400 MHz WB NMR spectrometer equipped with a 5 mm QNP probe (^{1}H/^{13}C/^{19}F/^{31}P). Typical experimental parameters were chosen as follows: spectral width 7.5 kHz, ^{19}F excitation pulse 18 µs, acquisition time 2 s, relaxation delay 2 s, number of scans 2048, proton decoupling using Waltz-16 with γB_1 = 1 kHz. All time domain data were processed with an exponential window function using a line broadening factor of 12-30 Hz.

^{19}F-resonances were referenced relative to external CCl_3F.

Sample preparation

Oligonucleotides (triethylammonium salts) together with the adequate amount of sodium phosphate buffer were lyophilized to dryness, and subsequently dissolved in H_2O/D_2O (9/1, v/v, 500 µL). All samples were heated to 90 °C for two minutes, then rapidly cooled to room temperature and equilibrated for one hour before measurements.

Titration experiments

Ligand binding was detected by acquiring standard ^{19}F-1D-NMR spectra using a 500 µl sample of 200 µM fluorine labeled 27 nt RNA sequence in 25 mM sodium phosphate buffer (pH 6.5) in presence and absence of various classes of aminoglycosides (tobramycin, neomycin B, paramomycin, ribostamycin, streptomycin, spectinonmycin). Aminoglycosides were added as 100 mM stock solutions in 25 mM sodium phosphate buffer (pH 6.5).

K_d determination

The affinity of a ligand to an RNA in a two site second order exchange is represented by the dissociation constant of the RNA-ligand complex $K_{d,}$ which is defined as

$$K_d = \frac{[RNA] \cdot [ligand]}{[RNA - ligand]}$$

with

[RNA] = concentration of free RNA

[ligand] = concentration of free ligand

[RNA-ligand] = concentration of RNA – ligand complex

The total ligand concentration is defined as

$$[L_0] = [ligand] + [RNA - ligand],$$

the total RNA concentration as

$$[R_0] = [RNA] + [RNA - ligand]$$

In the fast exchange regime the rate of equilibration between free and ligand-bound state is faster than the chemical shift separation $\Delta\delta$. Thereby, a single resonance, δ_{obs}, is observed at a weigthed average given by

$$\delta_{obs} = \delta_{RNA}\, p_{RNA} + \delta_{RNA\text{-}ligand}\, p_{RNA\text{-}ligand}$$

with

δ_{RNA} chemical shift of free RNA

p_{RNA} RNA mole fraction in free state

$\delta_{RNA\text{-}ligand}$ chemical shift of RNA in bound state

$p_{RNA\text{-}ligand}$ RNA mole fraction in bound state

The difference between the observed chemical shift and the shift value of the free RNA, $\Delta\delta$, is proportional to the mole fraction of the RNA in the ligand-bound state,

$$\Delta\delta = p_{RNA\text{-}ligand}(\delta_{RNA\text{-}ligand} - \delta_{RNA\text{-}ligand})$$

By expressing the mole fraction of the RNA – ligand complex in terms of the total RNA and ligand concentrations and K_d, the following equation is obtained

$$\Delta\delta = \frac{(\delta_{RNA-ligand} - \delta_{RNA})\{(R_0 + L_0 + K_d) - \sqrt{(R_0 + L_0 + K_d)^2 - 4R_0L_0)}\}}{2R_0}$$

The values of K_d and $\delta_{RNA\text{-}ligand}$ can be determined from the titration of the RNA aptamer with ligand by a non-linear least squares fitting of the chemical shift changes as a function of total ligand concentration $[L_0]$ to the previous equation.[3] This was done for the titration of the 27 nt double labeled RNA sequence and streptomycin, where the loop 2'-fluorine adenosine loop label showed broadening and a distinct downfield shift, indicating a well-behaved fast exchange ligand-RNA interaction.

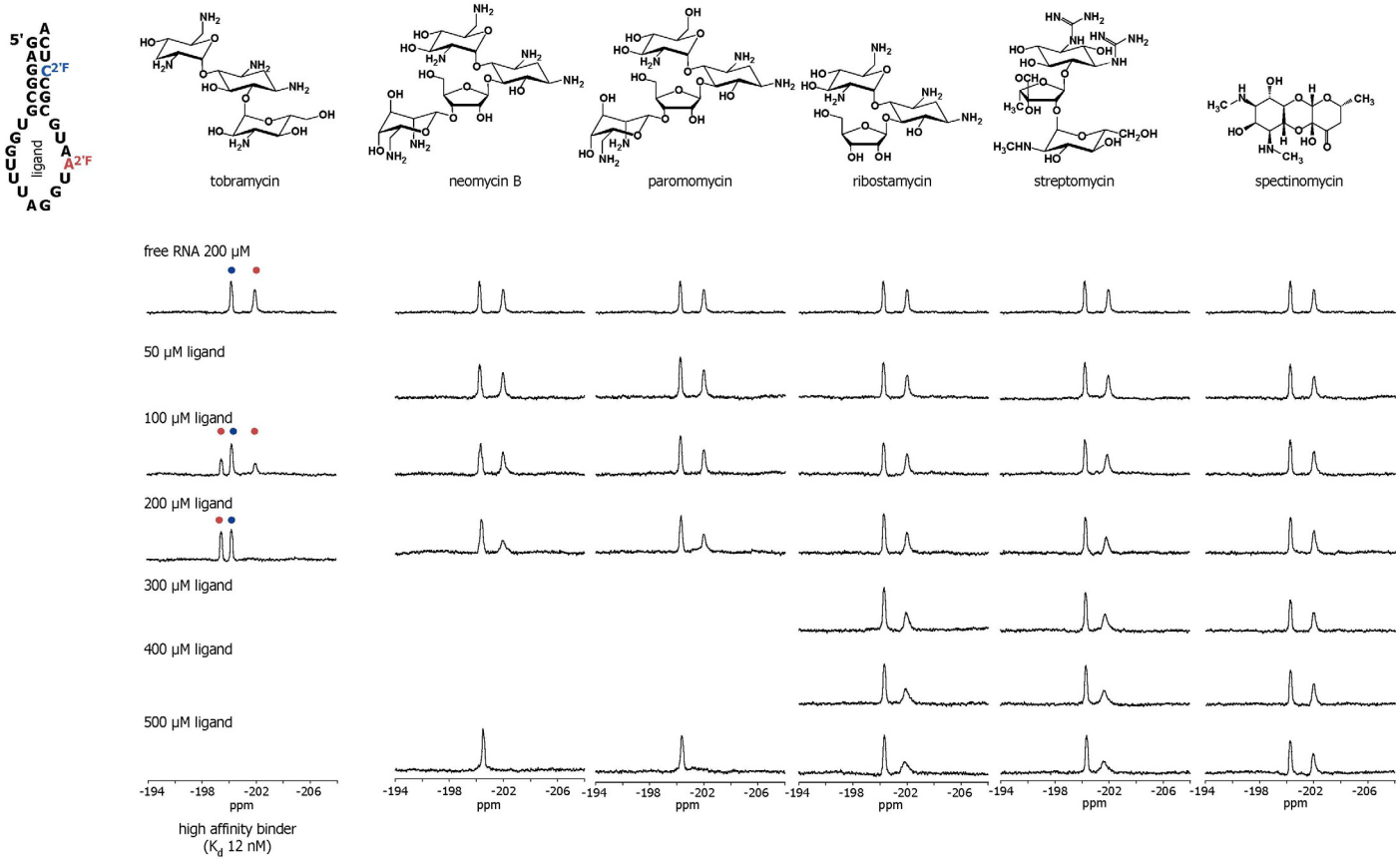
Supplementary Material References

1. Emsley, L.; Bodenhausen, G. Gaussian pulse cascades: new analytical functions for rectangular selective inversion and in-phase excitation in NMR. *Chem. Phys. Lett*. **1990**, *165*, 469-476.

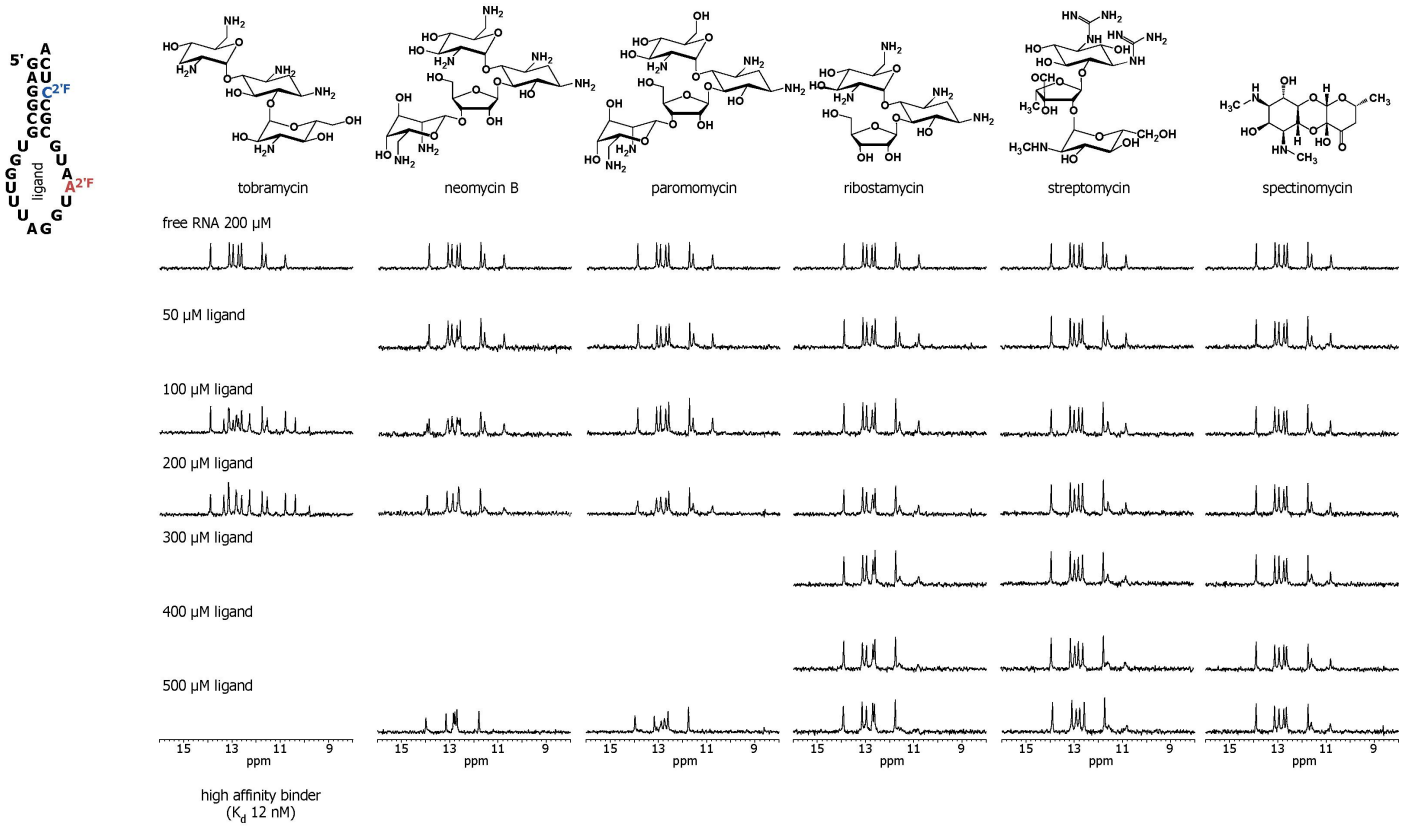
2. Geen, H.; Freeman, R. Band-selective radiofrequency pulses. *J. Magn. Reson*. **1991**, *93*, 93-141.

3. Lian, L.-Y., Roberts, G. C. K. Effects of chemical exchange on NMR spectra. In *NMR of Macromolecules* (Roberts, G. C. K., editor), Oxford University Press, Oxford, **1993**, p. 153.

2. ^{19}F-NMR spectroscopy of RNA – aminoglycoside complexes



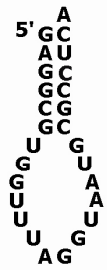
3. ^{1}H-NMR spectroscopy of RNA – aminoglycoside complexes

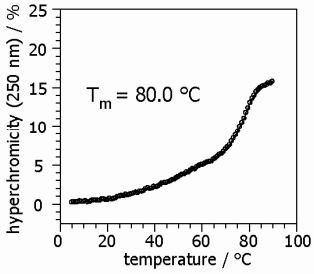


conditions: 200 µM RNA, 25 mM sodium phosphate buffer, pH 6.5, H_2O/D_2O 9/1 (v/v), 298 K

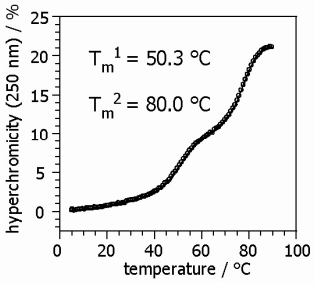
4. Melting curve analysis of native and fluorinated tobramycin aptamer sequence

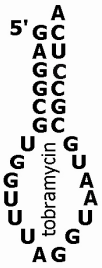
unmodifed 27 nt RNA aptamer sequence





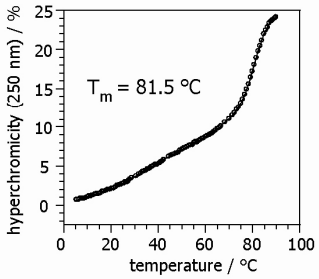
2 µM RNA



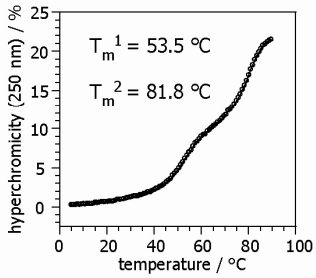


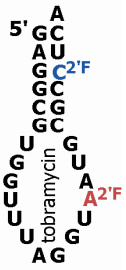
2 µM RNA, 3 µM tobramycin

fluorinated 27 nt RNA aptamer sequence



2 µM RNA

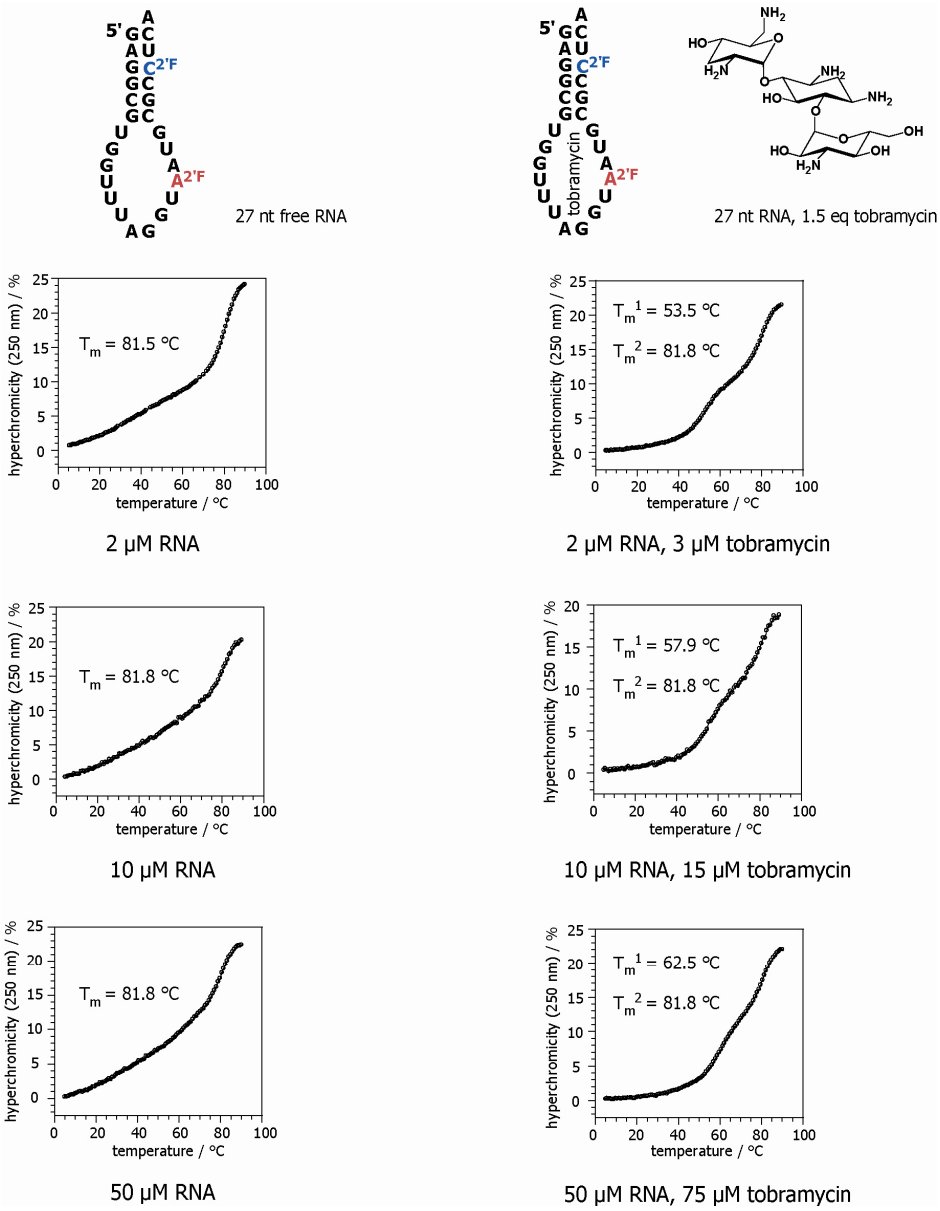




2 µM RNA, 3 µM tobramycin

conditions: 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0

5. Concentration dependence of melting points of free 27 nt fluorinated RNA and complexed 27 nt fluorinated RNA

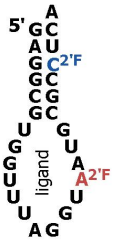


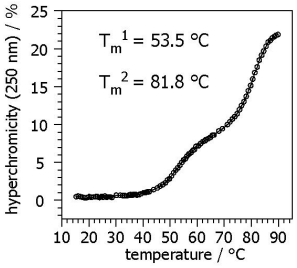
conditions: 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0

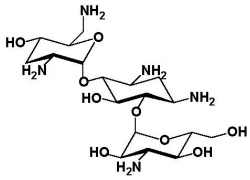
The high temperature melting transition is associated to the opening of the stem region, whereas the low temperature melting event, which is pronounced in the presence of tobramycin, is likely to be related to the dissociation of tobramycin accompanied by the opening of the base-pairing interactions within the loop region.

Assuming that the temperature dependence of the lower melting point is attributed to a bimolecular behavior of tobramycin dissociation, a ΔG^0_{off}–value of 57 kcal mol^{-1} can be determined. This result is in good agreement with the ΔG^0_{off}-value derived from the independently determined dissociation constant (K_d = 12 nM, ΔG^0_{off} = 45 kcal mol^{-1}, Y. Wang, R. R. Rando, *Chem. Biol.* **1995**, 2, 281-290).

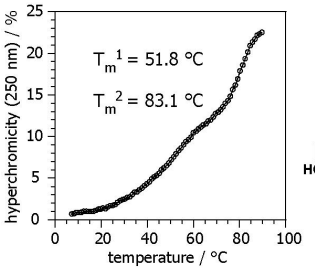
6. Melting curve analysis of RNA – aminoglycoside complexes

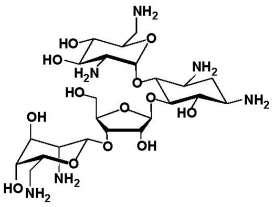




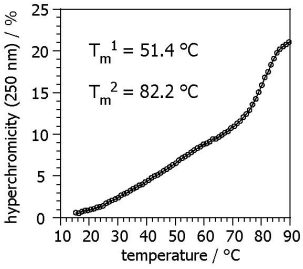


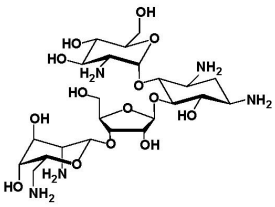
27 nt RNA, 1.5 eq tobramycin



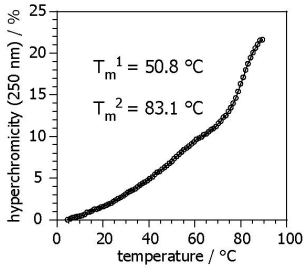


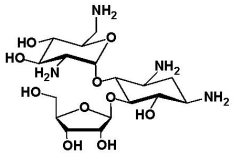
27 nt RNA, 1.5 eq neomycin B



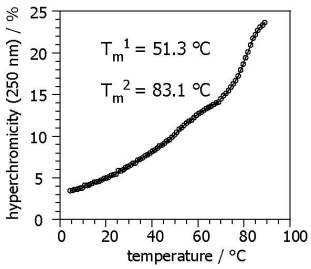


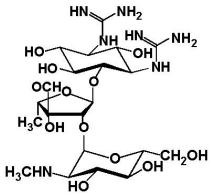
27 nt RNA, 1.5 eq paramomycin



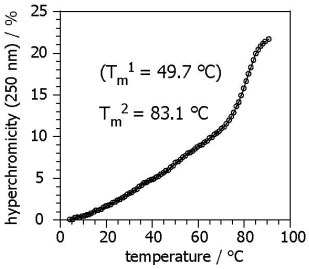


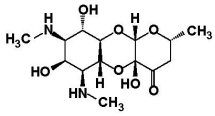
27 nt RNA, 1.5 eq. ribostamycin





27 nt RNA, 1.5 eq. streptomycin

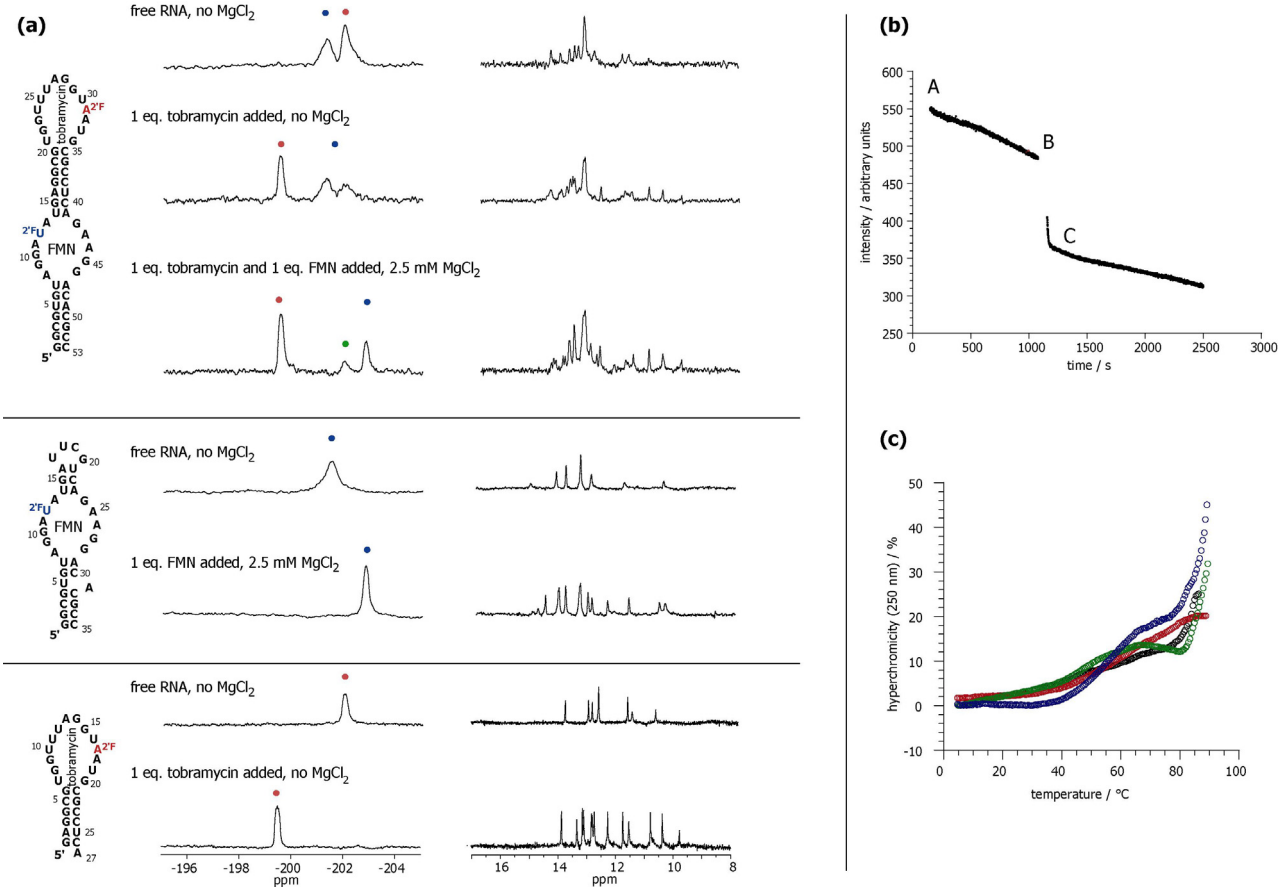




27 nt RNA, 1.5 eq. spectinomycin

conditions: 2 µM RNA, 3 µM aminoglycoside, 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0

7. 53 nt double aptamer sequence



(a) ^{19}F NMR spectrum and corresponding ^{1}H NMR spectrum (imino proton region), conditions: 200-300 µM RNA, 25 mM sodium phosphate buffer, pH 6.5, H_2O/D_2O 9/1 (v/v), 298 K

(b) Fluorescence spectroscopy based assay for FMN-RNA-binding

A: 2.5 µM FMN, 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0 (placed in a quartz-cuvette),

B: addition of the pre-formed complex of 53 nt double aptamer sequence (3.75 µM) and 1.5 eq. tobramycin (2.5 µM) and 2. 5 mM $MgCl_2$ to the quartz-cuvette,

C: decrease in FMN fluorescence intensity due to FMN binding to the 53 nt RNA aptamer sequence Photobleaching (note fluorescence decrease between point A and B) of FMN has an insignificant contribution to the signal decrease.

(c) Melting curve analysis of 53 nt sequence

black trace free RNA (T_m n. d. (> 90 °C)),

red trace 53 nt RNA, 1.5 eq. tobramycin (T_m^1 = 58. 5 °C, T_m^2 = 79.5 °C),

green trace 53 nt RNA, 1.5 eq. FMN (T_m^1 = approx. 55 °C (broad maximum), T_m^2 = n. d. (> 90 °C)),

blue trace 53 nt RNA, 1.5 eq. FMN, 1.5 eq. tobramycin (T_m^1 = 55.9 °C, T_m^2 = n. d. (> 90 °C))

conditions: 2 µM RNA, 150 mM NaCl, 10 mM Na_2HPO_4, 2.5 mM $MgCl_2$, pH 7.0, n.d. not determined